Growth Arrest Homeobox Gene

Background of the Invention

The leading cause of death in the United States and in most developed countries, is atherosclerosis. Atherosclerosis is a disease affecting the large and medium size muscular arteries such as the coronary or carotid arteries and the large elastic arteries such as the aorta, iliac, and femoral arteries. This disease causes narrowing and calcification of arteries. The narrowing results from deposits of substances in the blood in combination with proliferating vascular smooth muscle cells.

The deposits known as atherosclerotic plaques are comprised of lipoproteins, mainly cholesterol, proliferating vascular smooth muscle cells and fibrous tissue, and extra cellular matrix components, which are secreted by vascular smooth muscle cells. As the plaques grow, they narrow the lumen of the vessel decreasing arterial blood flow and weakening the effected arteries. The resulting complications potentially include a complete blockage of the lumen of the artery, with ischemia and necrosis of the organ supplied by the artery, ulceration and thrombus formation with associated embolism, calcification, and aneurysmal dilation. When atherosclerosis causes occlusion of the coronary arteries, it leads to myocardial disfunction, ischemia and infarction and often death. Indeed, 20-25% of deaths in the United States are attributable to atherosclerotic heart disease. Atherosclerosis also leads to lower extremity gangrene, strokes, mesenteric occlusion, ischemic encephalopathy, and renal failure, depending on the specific vasculature involved. Approximately 50% of all deaths in the United States can be attributed to atherosclerosis and its complications.

Present treatments for atherosclerosis include drugs and surgery, including ballon angioplasty. As a

result of angioplasty, vascular smooth muscle cells de-differentiate and proliferate and leading to leading to reocclusion of the vessel. These de-differentiated vascular smooth muscle cells deposit collagen and other matrix substances, that contribute to the narrowing of vessel. Vascular cells secrete growth factors such as platelet derived growth factor, which induces both chemotaxis and proliferation of vascular smooth muscle cells.

Many of the present drug therapies treat a predisposing condition such as hyperlipidemia, hypertension, and hypercholesterolemia, in an attempt to slow or halt the progression of the disease. Other drug therapies are aimed at preventing platelet aggregation or the coagulation cascade. Unfortunately, the drug treatments do not reverse existing conditions.

Surgical treatments include coronary artery bypass grafting, balloon angioplasty, or vessel endarterectomy which, when successful, bypass or unblock occluded arteries thereby restoring blood flow through the artery. The surgical treatments do not halt or reverse the progression of the disease because they do not affect smooth muscle cell proliferation and secretion of extra cellular matrix components.

The bypass surgeries, particularly the coronary bypass surgeries, are major, complicated surgeries which involve a significant degree of risk. The balloon angioplasty, while also a surgical procedure, is less risky. In balloon angioplasty, a catheter having a deflated balloon is inserted into an artery and positioned next to the plaque. The balloon is inflated thereby compressing the plaque against the arterial wall. As a result, the occlusion is decreased and increased blood flow is restored. However, the balloon angioplasty injures the arterial wall. As a result, the underlying vascular smooth muscle cells migrate to the intima, and synthesize and excrete extracellular matrix components eventually leading

to the reocclusion of the vessel. Of the estimated 400,000 coronary artery balloon angioplasties performed each year in the United States, 40% fail due to reocclusion requiring a repeat procedure or coronary bypass surgery. Bypass surgeries also have a significant rate of failure due to internal hyperplasia, which involves excessive proliferation of vascular smooth muscle cells at the sites of vascular anastamoses.

Attempts have been made to prevent reocclusion of vessels after balloon angioplasties in experimental animals. One approach has been to treat rat carotid arteries with antisense oligonucleotides directed against the c-myb gene following balloon angioplasty de-endothelialization. In vascular smooth muscle cells the The expression of the c-myb gene is up-regulated during the G1 to S transition of the cell cycle, and the activation of c-myb expression is required for further cell cycle progression. The antisense oligonucleotides to c-myb blocked smooth muscle cell proliferation following balloon angioplasty. However, the antisense oligonucleotides are applied in a pleuronic gel to the adventitia, that is, the exterior, rather than the lumen side of the affected vessel. Exposing the the exterior of the vessel requires additional surgery with its attendant risks, and is therefore not desirable.

It would be desirable to have a nonsurgical treatment, used in conjunction with balloon angioplasties to reduce vascular smooth muscle cell proliferation.

Summary of the Invention

A novel growth arrest homeobox gene has been discovered and the nucleotide sequences have been determined in both the rat and the human. The expression of the novel homeobox gene inhibits vascular smooth muscle cell growth. The growth arrest homeobox gene hereinafter referred to as the "Gax gene" and its corresponding proteins are useful in the study of vascular smooth muscle

cell proliferation and in the treatment of blood vessel diseases that result from excessive smooth muscle cell proliferation, particularly after balloon angioplasty.

Brief Description of the Drawings

Figure 1 is the nucleotide sequence of rat Gax gene with the predicted amino acid sequence listed below the nucleotide sequence. The homeobox is indicated by a box, and the CAX nucleotide repeat, where X is ether cytosine or guanine, is underlined. A polyadenylation signal is in boldface and italics. Putative consensus sites are indicated as follows: for phosphorylation by protein kinase C, circles; for cyclic AMP (cAMP)-dependent protein kinase, squares; for casein kinase II, diamonds; and for histone H1 kinase, triangles. Residues which could potentially be a target for either cAMP-dependent protein kinase or protein kinase C are both circled and boxed.

Figure 2 is the map of mouse chromosome 12 showing the location of the Gax gene;

Figure 3 is the nucleotide sequence of human Gax gene with the predicted amino acid sequence listed below the nucleotide sequence;

Figure 4 is a map of human Gax gene showing how the separately cloned fragments were joined and oriented in the plasmid, Bluescript IISK+;

Figure 5A is a northern blot showing Gax RNA levels in vascular smooth muscle cells in response to 10% fetal calf serum after 4, 24, and 48 hours; lane Q is RNA from quiescent cells; GAPDH is rat glyceraldehyde 3-phosphate dehydrogenase;

Figure 5B is a northern blot showing Gax RNA levels and Hox 1.3 RNA levels in vascular smooth muscle cells in response to 10 ng/ml human platelet derived growth factor at 0.25, 0.5, 1, 2, and 4 hours, lane Q is RNA from quiescent vascular smooth muscle cells;

Figure 6 is a graph of changes in relative Gax mRNA levels in vascular smooth muscle cells in response to

10% fetal calf serum and 10 mg/ml of the PDGF isoforms; the circles represent PDGF-AA, the squares represent PDGF-BB, the diamonds represent fetal calf serum, and the triangles represent PDGF-AB;

Figure 7 is a graph showing ^{3}H-thymidine uptake in vascular smooth muscle cells at various times after stimulation with fetal calf serum and PDGF isoforms; the circles represent PDGF-AA, the triangles represent PDGF-AB, the squares represent PDGF-BB, the diamonds represent fetal calf serum, and the solid squares represent no mitogen;

Figure 8 is a graph showing relative Gax mRNA levels in vascular smooth muscle cells in response to varying doses of PDGF-AB, represented by triangles, and PDGF-BB, represented by squares;

Figure 9 is a graph showing relative Gax mRNA levels in vascular smooth muscle cells in response to varying doses of fetal calf serum;

Figure 10 is a graph showing relative Gax mRNA levels in vascular smooth muscle cells in response to fetal calf serum withdrawal;

Figure 11 is a dose response curve showing % inhibition of growth in vascular smooth muscle cells in response to varying doses of microinjected GST-Gax protein;

Figure 12 is a graph showing percent inhibition of mitogen induced DNA synthesis in vascular smooth muscle cells in response to: ras (Leu-61) protein; ras (Leu-61) protein in combination with the GST-Gax protein; GST-Gax protein; and the GST;

Figure 13 is a graph showing percent inhibition of vascular smooth muscle cell entry into S phase by microinjected GST-Gax protein over time and the ^{3}H-thymidine uptake over the same time period;

Figure 14 is a graph showing the ratio of the Gax mRNA to glyceraldehyde-3-phosphate dehydrogenase designated "G3" level from normal vascular tissue and times following acute blood vessel injury.

Detailed Description of the Invention

A novel gene, the Gax gene, has been discovered, the expression of which inhibits vascular smooth muscle cell growth. The Gax gene and the protein it encodes, referred to herein as the "Gax protein" are useful in the study of vascular smooth muscle cell proliferation and in inhibiting smooth muscle cell proliferation. The inhibition of vascular smooth muscle cell proliferation, particularly by genetic therapy, is also useful in the treatment of vascular diseases associated with excessive smooth muscle cell proliferation.

Nucleotide sequences, such as the Gax gene or portions therof, or mRNA are administered to the vascular cells, preferably during a balloon angioplasty procedure, to inhibit the proliferation of vascular smooth muscle cells. The nucleotide sequences are delivered, preferably to the interior of the vessel wall during balloon angioplasty procedure preferably by a perforated balloon catheter. Genes are transfered from vectors into vascular smooth muscle cells in vivo where the genes are expressed. Suitable vectors and procedures for the transfer of nucleotides are found in

Nabel, E. G., et al. "Site-Specific Gene Expression in Vivo by Direct Gene Transfer into the Arterial Wall" (1990) Science Vol. 249, pp. 1285-1288, which is incorporated herein by reference. Specialized perforated balloon catheters which deliver nucleotide sequences to vessel walls employing viral and non-viral vectors are used for delivery of nucleotide sequences and a description of the catheter's structure and use may be found in Flugelman M.Y., et al. "Low Level In Vivo Gene Transfer Into the Arterial Wall Through a Perforated Balloon Catheter" Circulation, Vol. 85, No. 3, pp. 1110-1117 (March 1992) which is incorporated herein by reference.

Genetic therapy, preferably by the over expression of the Gax gene, restores the proliferating vascular smooth muscle cells to a more normal phenotype,

thus preventing or reducing the smooth muscle proliferation that is associated with the formation of the atheromatous plaque and with internal arterial thickening following balloon angioplasty. In addition to preventing or reducing the reocclusion of the vessel, such genetic therapy decreases the risks associated with additional surgeries. Also, the Gax proteins or portions thereof, are administered to vascular cells preferably employing the perforated catheter, to inhibit the proliferation of vascular smooth muscle cells.

The molecular control of cellular proliferation is not well understood. A class of genes, known as Homeobox genes, encode a class of transcription factors which are important in embryogenesis, tissue specific gene expression and cell differentiation. The homeobox genes share a highly conserved 183 nucletide sequence that is referred to as the "homeobox". The homeobox encodes a 61 amino acid helix-turn-helix motif that binds to adenine and thymine rich gene regulatory sequences with high affinity. Several vertebrate homeobox proteins have been shown to be transcription factors required for expression of lineage-specific genes. The tissue-specific transcription factors bind to DNA and repress or induce groups of subordinate genes. Many, but not all of these homeobox genes are located in one of four major clusters known as Hox clusters, designated Hox-1, Hox-2, Hox-3 and Hox-4. The Hox genes are expressed in the developing embryo, in distinct overlapping spatial patterns along the anterior-posterior axis which parallels the Hox gene order along the chromosome. Homeobox transcription factors control axial patterning, cell migration and differentiation in the developing embryo and are involved in the maintenance of tissue specific gene expression in adult organisms.

A new homebox gene has been discovered, isolated and sequenced in both the rat and human. This new gene is a growth arrest specific homeobox gene and is referred to herein as the "Gax gene". The expression of the Gax gene

is restricted to the cardiovascular system, and in particular, to vascular smooth muscle cells where it functions as a negative regulator of cell proliferation.

Isolation of the rat Gax cDNA

An adult rat aorta cDNA library in λ ZAP, from Stratagene, was screened with a 64-fold degenerate 29-mer oligonucleotide containing three inosine residues directed at the most highly conserved region of the *antennapedia* homeodomain (helix 3), with the following sequence, where I represents inosine:

5'-AA(A/G)ATITGGTT(T/C)CA(A/G)AA(C/T)(A/C)GI(A/C)GIATGAA-3'.

Recombinant phage colonies in *Escherichia coli* were adsorbed in duplicate to nitrocellulose membranes and hybridized at 42°C with this oligonucleotide end labeled with (γ-32P)ATP in a mixture containing 0.5 M sodium phosphate at pH 7.0, 7% sodium dodecyl sulfate, 1 mM EDTA, and 1% bovine serum albumin. The filters were washed with a final stringency of 0.5 x SSC (1 x SSC in 150 mM NaCl with 15 mM sodium citrate at pH 7.0)-0.1% sodium dodecyl sulfate at 42°C and exposed to X-ray film. Thirteen positive signals were isolated and rescreened until the clones were plaque purified. The plasmids containing the clones in λ ZAP vector were then excised by the protocol recommended by the manufacturer and sequenced on both strands with sequenase version 2.0 from United States Biochemicals. From 500,000 plaques, 13 positive clones were isolated, 12 of which contained homeodomains. Nine of the isolated clones were derived from previously described homeobox genes: Hox-1.3, Hox-1.4, Hox-1.11, and rat homeobox R1b. However, three clones represented the cDNA designated herein as the "Gax" gene. Homology searches were performed via the GenBank and EMBL data bases, version 73, by using the BLAST algorithm (4).

Nucleotide Sequence of the rat Gax Gene

The nucleotide sequence of the rat Gax gene is shown in Figure 1. The cDNA encoding Gax is 2,244 base

pairs in length, which corresponds to the size of the Gax transcript, that is the Gax mRNA, which is about 2.3 to 2.4 kb as determined by Northern blot analysis. The Gax cDNA has an open reading frame from nucleotide residues 197 to 1108 beginning with an in-frame methionine that conforms to the eukaryotic consensus sequence for the start of translation and is preceded by multiple stop codons in all three reading frames. The open reading frame of the cDNA predicts a 33.6-kDa protein containing 303 amino acids with a homeodomain from amino acid residues 185 to 245, as shown in Figure 1. To confirm that this cDNA was capable of producing a protein product, the Gax open reading frame was fused in frame to the pQE-9 *E. coli* expression vector, from Qiagen, Inc., Chatsworth, California and expressed in bacteria according to Hochuli, E., *et. al.* (1988) "Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent" Bio/Technology Vol. 6, pp. 1321-1325. *E. coli* containing this plasmid expressed a new phosphorylated protein of about 30 to about 36 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and extracts from these *E. coli* cells displayed a weak binding activity for the adenine and thymine rich, MHox-binding site in the creatine kinase M enhancer.

The cDNA encoding the rat Gax gene also contains a long 3'-untranslated region, from bases 1109 to 2244, with a polyadenylation signal at base 2237, as shown in Figure 1. The region between amino acids 87 and 184 contains 23 serine amino acids out of 88 amino acids and 10 proline amino acids out of 88 amino acids and contains several consensus sequences for phosphorylation by protein kinases. Gax also possesses a structural feature which is also found in several transcription factors, including homeodomain proteins, known as the CAX or Opa transcribed repeat. The Opa transcribed repeat encodes a stretch of glutamines and histidines; in the rat Gax gene it encodes 18 residues, of which 12 are consecutive histidines. This

motif is shared by other transcription factors, such as the zinc finger gene YY-1, as well as by several homeobox genes, including H2.0, HB24, ERA-1 (Hox-1.6), Dual bar, and Tes-1. The Gax protein may require post-translational modifications for full activity, modifications that bacterially produced proteins do not undergo. Since the Gax protein has multiple consensus sites for phosphorylation by protein kinases, it is possible that its activity is activated or otherwise modulated by phosphorylation at one or more of those sites.

The Gax Gene Maps to a Chromosome 12 of the Mouse Genome

Gax is located on chromosome 12 as shown in Figure 2 of the mouse and is not a part of the Hox-1, Hox-2, Hox-3, or Hox-4 gene clusters, which are located on chromosomes 6, 11, 15, and 2, respectively, McGinnis, W., and R. Krumlauf, (1992) "Homeobox genes and Axial Patterning" Cell, Vol. 68, pp. 283-302. Also Gax does not cosegregate with any other homeobox genes previously mapped in the interspecific backcross. A comparison was done of the interspecific map of chromosome 12 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations using GBASE, a computerized data base maintained at The Jackson Laboratory, Bar Harbor, Maine. The Gax gene mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus.

The mouse chromosomal location of the Gax was determined by interspecific backcross analysis using progeny generated by mating (C57BL/6J *x Mus spretus*) F_1 females and C57BL/6J males. The C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms with a rat cDNA Gax probe. The probe, a 1,155-bp rat cDNA clone, was labeled with (α-^{32}P)dCTP by using a random prime labeling kit from Amersham and washing was done with a final stringency of 0.2 x SSCP (34)-0.1% sodium dodecyl sulfate, 65°C. A major

fragment of 4.2 kb was detected in *Hinc*II-digested C57BL/6J DNA, and major fragments of 3.6 and 2.7 kb were detected in *Hinc*II-digested *M. spretus* DNA. The 3.6-kb and 2.7-kb *M. spretus Hinc*II restriction fragment length polymorphisms were used to monitor the segregation of the Gax locus in backcross mice. Recombination distances were calculated by using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

The mapping results indicated that the mouse Gax gene is located in the proximal region of mouse chromosome 12 linked to neuroblastoma *myc*-related oncogene 1 (*Nmyc-1*), the laminin B1 subunit gene (*Lamb-1*), a DNA segment, chromosome 12, the Nyu 1 gene (*D12Nyu1*), and the β-spectrin gene (*Spnb-1*). The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are as follows: centromere-*Nmyc-1*-19/193-*Lamb-1*-9/166-Gax-10/166-*D12Nyu1*-19/185-*Spnb-1*. The recombination frequencies, expressed as genetic distances in centimorgans ± the standard error, are as follows: *Nmyc-1*-9.8 ± 2.2-*Lamb-1*-5.4 ± 1.8-Gax-6.0 ± 1.9-*D12Nyu1*-10.3 ± 2.2-*Spnb-1*.

Gax Gene Expression in Rat Tissue

It has been discovered that the Gax transcript is largely confined to the cardiovascular system, including the descending thoracic aorta, where it is expressed at higher levels than in other tissues, and the heart. Gax gene expression was also detected in the adult lung and kidney where it is found in mesangial cells. No Gax gene expression was detected in the brain, liver, skeletal muscle, spleen, stomach, or testes, nor was expression detected in the intestine or pancreas. In contrast, the Gax gene was more widely expressed in the developing embryo, with the transcript detectable in the developing cardiovascular system, multiple mesodermal tissues, and some ectodermal tissues.

The 2.3-kb to 2.4-kb Gax RNA transcript was detected in smooth muscle cells cultured from adult rat aorta, consistent with the in situ hybridization findings and the fact that Gax was originally isolated from a vascular smooth muscle library. The Gax transcript was also detected in rat vascular smooth muscle cells transformed by simian virus 40. However, no Gax gene expression was detected in either of two cell lines derived from embryonic rat aortic smooth muscle, A7r5 and A10. The Gax transcript was also not detected in NIH 3T3 fibroblasts, or human foreskin fibroblasts. The Gax transcript was not detected in the skeletal muscle cell line C2C12. A relatively high level of Gax gene expression was detected in cultured rat mesangial cells. Mesangial cells share many similarities to vascular smooth muscle cells, both structurally and functionally, and proliferate abnormally in renal diseases such as glomerulonephritis and glomerulosclerosis.

Isolation of the Human Gax cDNA

The nucleotide sequence of the human Gax gene coding sequence is shown in Figure 3. Approximately 1 x 10^6 plaques from a human genomic library in λFixII available from Stratagene were screened by conventional methods with a random primed EcoRI/BstXI fragment encompassing nucleotides 485-1151 of the rat Gax cDNA. Two clones contained the second exon of human Gax gene, having 182 base pairs. Using this coding information, the rest of the coding region was cloned by polymerase chain reaction methods.

Reverse transcriptase and polymerase chain reaction techniques were used to clone the 3' end of the human cDNA. The template was whole human RNA isolated from human internal mammary artery isolated by TRI reagent from Molecular Research Center, Inc. The following reagent concentrations were used in the reverse transcriptase reaction: 1μg of total internal mammary artery RNA; 50 mM Tris-HCl pH 8.5; 30 mM KCl; 8 mM $MgCl_2$; 1 mM DTT; 20 units

RNAsin from Boehringer Mannheim; 1mM each of dATP, dTTP, dGTP, and dCTP; 0.5 μg random hexamers from Boehringer Mannheim; and 40u of AMV reverse transcriptase from Boehringer Mannheim, in a total volume of 20 μL. This was incubated for 1 hour at 42°C, heat inactivated, and then stored at -80°C before use. An initial amplification of 10% of the reverse transcriptase reaction was performed with just the sense oligonucleotide primer, known as "H2" and Ampliwax™ PCR Gem 100 beads Perkin Elmer in a "hot start" procedure according to the directions of the manufacturer. The following reagent concentrations were used: 50 mM KCL; 10 mM Tris-HCl at pH 8.3; 1.5 mM $MgCl_2$; 1 mg/mL gelatin; 0.2 mM each of dATP, dTTP, dGTP, and dCTP; 0.1 μM primer(s); and 2.5 units of Taq polymerase from Boehringer Mannheim or Perkin Elmer in a volume of 100μL (these conditions were used thereafter unless noted). The cycling protocol was as follows: 94°C for two minutes, then 30 cycles of 94°C for 30 seconds, 45°C for 1 minute, and 72°C for 1 minute. A second amplification was then performed on 10% of the primary reaction products using the H2 primer and a degenerate antisense oligonucleotide primer known as "P2B" against the carboxy terminal peptide. The cycling parameters were: 94°C for two minutes followed by 30 cycles of 94°C for 30 seconds, 40°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute. A product was observed of the correct size and following purification by Glass Fog from Bio101, on 2% Biogel agarose from Bio101 was blunt sub-cloned into EcoRV digested BluescriptII SK+ vectors from Stratagene and sequenced to high resolution by Sequenase 2.0 from universal primers from United States Biochemical. Five individual clones were sequenced to eliminate any spurious Taq polymerase errors.

The 5' end of the human coding region was amplified using an anchored polymerase chain reaction kit, available under the tradename "5'-Amplifinder RACE" from Clonetech according to the manufacturer's instructions. This method uses single stranded RNA ligase to ligate an

anchor oligonucleotide onto the 3' end of appropriately primed first strand cDNA. Templates used were either human heart polyA+ RNA obtained from Clonetech or polyA+ RNA isolated from primary cultures of human vascular smooth muscle cells obtained from Clonetics. The polyA+ RNA from cultured vascular smooth muscle cells was purified with RNAzol B from Biotecx using batch chromatography on Oligo-dT latex beads from Qiagen. Both templates yielded amplified cDNAs and specific subclones were chosen solely by size. First strand RNA templates were prepared by either specific priming or priming with random hexamers from Boehringer Mannheim. In general, the specific primed templates yielded longer clones but could not be used for multiple step wise amplification of the rest of the coding region.

Amplification from anchored templates using the sense anchor primer and appropriate antisense specific primers was accomplished using ampliwax beads from Perkin Elmer and "hot start" polymerase chain reaction using the same reaction conditions as above, but with 0.2μM primers in a total volume of 50 μL. The cycling protocol was as follows: 94°C for 2 minutes then 30 cycles of 94°C 45 seconds, 60°C 45 second, and 72°C for 1.5 minutes, followed by a final extension of 72°C for 10 minutes. Following a primary amplification, aliquots (10-20%) of the reactions were run out on 2% Biogel agarose from Bio101 and size selected. After purification by glass fog from Bio101, 1-10% of the elutes were reamplified (2°), usually with a nested primer. Products were observed at this point and purified by glass fog as before and sequenced directly using a thermal cycling kit from New England Biolabs. Once the products were confirmed they were sub-cloned as described above. Between 5 to 8 individual clones from each of three sequential amplifications were sequenced to eliminate spurious Taq polymerase errors and appropriate clones chosen for the finished molecule. A summary of the primer pairs sense/antisense used to amplify the complete

coding region follows:

position Clone #	Source of Template	1°	2°	5'-3'
6	dN6 primed IMA whole RNA	H2	H2/P2B	699-941
23	H2R primed Heart polyA+RNA	AP/H2R	AP/H3	231-698
117	dN6 primed VSMC poly A+RNA	AP/H6	AP/H6	119-230
131	dN6 primed VSMC poly A+RNA	AP/H6	AP/H7	1-118

Clones were pieced together 3'-5' as follows: fragments 6 and 23 share engineered BglII sites; fragments 23 and 117 share a native SfaNI site; fragment 117 has a native NcoI site which is compatible with an engineered BspHI site in fragment 131. Both engineered sites have a single base change in the wobble base of leucine codons, as noted on the final sequence as shown in Figure 3. Once assembled the molecule was excised by digestion with EcoRI and HindIII. The map in Figure 4 shows the molecule and its

orientation.

TABLE 1

Primers Used to Amplify Human Gax gene

Primer	Sequence 5'-3'
P2B	TCA,IA(G/A),(G/A)TG,IGC,(G/A)TG,(T/C)TC
H2	GCGCGC(AGATCT)CAC,TGA,AAG,ACA,GGT,AAA
H2R	TT,TAC,CTG,TCT,TTC,AGT,GAG
H3	GCGCGC(AGATCT)AG,ATT,CAC,TGC,TAT,CTC,GTA
H6	GCGCGTGCCCCCTCTGATG,CTG,GCT,GGC,AAA,CAT,GT
H7	GCGCGC(TCTTGA)AGG,GCG,AGA,GAG,GAT,TGG,GA
AP	CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG
Anchor	GGAGACTTCCAAGGTCTTAGCTATCA(CTTAAG)CAC

Engineered enzyme sites are bracketed.

The Gax gene maps to a novel locus on Chromosome 7 in the human genome

To determine the map location of Gax in the human genome, a 16.5 kilobase pair fragment of the human genomic Gax gene in λFix II from Stratagene was purified with a Qiagen purification column according to the directions of the manufacturer, and it was labeled with biotin 11-dUTP by nick translation. Metaphase spreads of normal human lymphocytes were prepared according to the methods of Fan, Y., Proc. Natl. Acad. Sci. (USA) Vol. 87, pp. 6223-6227 (1990). Fluorescence in situ hybridization and immunofluorescence detection were performed according to the methods of Pinkel, D., et. al., Proc. Natl. Acad. Sci. (USA) Vol. 83, pp. 2934-2938 (1986) and Testa, J..R., et al. Cytogenet. Cell. Genet. Vol. 60, pp. 247-249 (1992). Chromosome preparations were stained with diamidino-2-phenylindole and propidium iodide according to Fan, Y.S., et. al., Proc. Natl. Acad. Sci. (USA) Vol. 87, pp.6223-6227 (1990).

Forty metaphase spreads were examined with a Zeiss Axiophot fluorescence microscope, and fluorescent signals were detected on the short arm of chromosome 7 in

34 of these spreads. All signals were located at p15-->p22, with approximately 70% of the signals at 7p21. Based on these data, Gax is the only homeoprotein known to map to this locus.

Gax gene expression is down-regulated in cultured vascular smooth muscle cells upon mitogen stimulation

It has been found that the Gax gene is expressed in quiescent vascular smooth muscle cells. Since platelet derived growth factor hereinafter also referred to as "PDGF" and other growth factors regulate vascular smooth muscle proliferation and differentiation, differences in Gax gene expression in response to PDGF and other mitogens such as fetal calf serum were examined in cultured vascular myocytes.

Cultures of rat smooth muscle cells were obtained from the media of aortas isolated from adult male Sprague-Dawley rats. Cells were seeded onto dishes in medium containing a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 and supplemented with 10% newborn calf serum. Once established, the cells were maintained at 37° C in a humidified atmosphere of 5% carbon dioxide, and subcultured within three days after reaching confluence. Vascular smooth muscle cells were labeled with monoclonal antibodies to smooth muscle α-actin from Sigma Chemical Co. to verify identity.

The cultured cells were exposed to various mitogens as discussed below. The cells were then harvested and the total mRNA was extracted. The total RNA from rat cultured cells was prepared by the guanidine thiocyanate method according to Chomcynzski, P., and N. Sacchi, (1987) "Single-step Method of RNA Isolation by Acid Guanidinium Thiocyanate-phenol-chloroform Extraction" Anal. Biochem. Vol. 162, pp. 156-159, fractionated on 1.2% agarose gels containing formaldehyde, and blotted onto nylon membranes. The RNA from cultured cells was separated on 30-cm gels for transcript size determination and on 10-cm gels for other

studies. Hybridizations were carried out at 65°C in buffer containing 0.5 M sodium phosphate at pH 7.0, 7% sodium dodecyl sulfate, 1 mM EDTA, and 1% bovine serum albumin, using a cDNA probe labeled by random priming consisting of a truncated Gax cDNA lacking the 5' end and the CAX repeat, where the X may be cytosine or guanine. Probes for Hox-1.3 and Hox-1.4 consisted of the cDNAs isolated from the rat aorta library, and the probe for Hox-1.11 consisted of the *Dra*I-*Eco*RI fragment of its cDNA. The blots were washed with a final stringency of 0.1 to 0.2 x SSC-0.1% sodium dodecylsulfate at 65°C. After the probings with the homeobox probes were complete, the blots were rehybridized with a probe to rat glyceraldehyde 3-phosphate dehydrogenase hereinafter also referred to as "GAPDH," to demonstrate message integrity. Gax mRNA and GAPDH mRNA were quantified with a Molecular Dynamics model 400S PhosphorImager to integrate bank intensities, or by scanning densitometry of autoradiograms. In all quantitative comparisons of Gax mRNA levels between experimental groups, Gax mRNA levels were normalized to the corresponding GAPDH level determined on the same blot, to account for differences in RNA loading.

Time Course of GAX Down-regulation in Cultured Vascular Smooth Muscle Cells

Rat vascular smooth muscle cells, grown to a greater than about 90% confluence, were placed in low-serum medium containing 0.5% calf serum for 3 days, to induce quiescence. At this time, the medium was removed from the cells and replaced with fresh medium containing either 10% fetal calf serum or 10 ng/ml platelet derived growth factor from human platelets. The cells were then incubated for the various times in the presence of either the fetal calf serum or the PDGF. As a control, quiescent cells were incubated with fresh serum-free medium alone. The cells exposed to PDGF were harvested at 0.25, 0.5, 1, 2, and 4 hours, and the cellular RNA isolated. The cells exposed to

human fetal calf serum were harvested at 4, 24, and 48 hours. The Gax and the Hox mRNA levels were determined by Northern blot analysis. Typical results are shown in Figures 5A and 5B.

A rapid down-regulation, that is a reduction in the amount of Gax mRNA, occurred in the vascular smooth muscle cells when they were stimulated with either fetal calf serum or PDGF as shown in Figures 5A and 5B. The down-regulation ranged from 5- to nearly 20-fold, depending on the mitogen used and the experiment. The down-regulation typically occurred within 2 hours after stimulation with fetal calf serum or PDGF, and was maximal at 4 hours. Gax mRNA transcript levels began to recover significantly by approximately 24 hours and approached baseline between 24 and 48 hours after stimulation. The rate of recovery varied with the magnitude of the initial down-regulation and the individual cell culture preparations. While PDGF isolated from human platelets caused a rapid down-regulation of Gax, it had little or no effect on Hox-1.3 mRNA levels. Neither fetal calf serum nor any of the three isoforms of PDGF showed any effect on the transcript levels of Hox-1.3, Hox-1.4, or Hox-1.11, homeobox genes which were also isolated from the vascular smooth muscle library.

Magnitude of Gax Down-regulation Correlates With Potency of Mitogen

PDGF is a homodimer or heterodimer made of various combination of two chains, A and B. Thus, there are three isoforms of PDGF; PDGF-AA; PDGF-AB; and PDGF-BB; and they have differing potencies for stimulating DNA synthesis in rat vascular smooth muscle cells. The PDGF-AA, PDGF-AB and PDGF-BB were compared for their effect on Gax expression. Quiescent rat vascular smooth muscle cell received 10 ng/ml of either PDGF-AA, PDGF-AB, PDGF-BB, or 10% fetal calf serum. After 0, 1, 2, 4 and 8 hours the cells were harvested and the Gax mRNA level determined. The results are shown in Figure 6.

As shown in Figure 6, PDGF-AA did not down-regulate Gax gene expression in vascular smooth muscle cells, whereas the PDGF-AB and PDGF-BB isoforms, and the fetal calf serum reduced Gax gene expression approximately 10-fold by 4 hours. The greatest down-regulation occurred with the fetal calf serum followed by that with PDGF-BB and PDGF-AB.

To determine whether the extent of Gax gene down-regulation correlated with the potency of the mitogen used to stimulate the vascular smooth muscle cells, the ability of the three PDGF isoforms and fetal calf serum to stimulate DNA synthesis was measured by ^{3}H-thymidine uptake. Quiescent vascular smooth muscle cells were stimulated with one of the three PDGF isoforms, at 10 mg/ml, or 10% fetal calf serum. Then 5 μCi/ml ^{3}H-thymidine was added to the cultures for 1 hour at various time points as shown in Figure 7. The cells were harvested and the ^{3}H-thymidine uptake was measured. The results are shown in Figure 7.

The PDGF-AA at 10 ng/ml, which was ineffective in causing Gax gene down-regulation, only weakly stimulated DNA synthesis as shown in Figure 7. PDGF-AB and PDGF-BB both stimulated cell proliferation as measured by ^{3}H-thymidine uptake at 15 hours. However, the fetal calf serum which was most effective at down regulating Gax gene expression, was also the most effective mitogen, that is it demonstrated the greatest ^{3}H-thymidine uptake.

Down-regulation of the Gax gene is Dependent on the Dose of the Mitogen

Dose-response experiments were conducted by stimulating quiescent vascular smooth muscle cells with either PDGF-AB, PDGF-BB or fetal calf serum at varying doses as shown in Figures 8 and 9. The effects on Gax mRNA levels were measured at 4 hours after mitogen stimulation. The results are shown in Figures 8 and 9.

As shown in Figure 8, the dose response curves reveal that the 50% effective dose for Gax gene down-

regulation 4 hours after PDGF-AB stimulation is between 4 and 8 ng/ml. The 50% effective dose for Gax gene down regulation 4 hours after PDGF-BB stimulation is between 2 and 5 ng/ml. The 50% effective dose for Gax down regulation 4 hours after fetal calf serum is approximately 1%, as shown in Figure 9. Furthermore, 10% fetal calf serum suppresses Gax mRNA levels nearly 20-fold at 4 hours, an effect larger than that of a maximal stimulatory dose of PDGF-BB (30 ng/ml), which has a 10-fold effect, or of PDGF-AB, which has a less than 8-fold effect, as shown in Figure 6. Thus, the down-regulation of Gax gene induced by either fetal calf serum or the different isoforms of PDGF correlates well with their abilities to stimulate DNA synthesis as measured by ^{3}H-thymidine uptake.

The Gax gene down-regulation is sensitive to low levels of mitogen stimulation, which cause a significant decrease in Gax mRNA levels. As shown in Figure 9, stimulation of quiescent rat vascular smooth muscle cells with 1% fetal calf serum caused a 40% decrease in Gax mRNA levels after 4 hours. However, such stimulation increased ^{3}H-thymidine uptake less than two-fold over that observed in quiescent vascular smooth muscle cells (data not shown). Treatment with PDGF-BB at doses as low as 2 ng/ml, also caused a detectable decrease in the Gax mRNA level.

<u>Gax Expression is Up-regulated or Induced when Synchronously Growing Cells Are Deprived of Serum</u>

Sparsely plated vascular smooth muscle cells were grown in a medium containing 20% fetal calf serum, and then placed into serum free medium. The RNA was harvested at various times from 0 to 25 hours. The total mRNA was extracted and subjected to Northern Blot Analysis, then the mRNA transcript of Gax was quantified.

As shown in Figure 10, the expression of the Gax gene was induced fivefold in vascular smooth muscle cells within 24 hours after the rapidly growing cells were placed in the serum-free medium. Thus, expression of Gax gene is

regulated by the growth state of the cell, and its down-regulation is a prominent feature of the G_0/G_1 transition in these cells.

Gax Protein Inhibits Mitogen-Induced S Phase Entry in Vascular Smooth Muscle Cells

Production of Recombinant Proteins

To determine whether Gax gene exerts a negative control on cell growth in vascular smooth muscle cells, Gax gene was expressed as a glutathione S-transferase (hereinafter also referred to as "GST") fusion protein in bacteria and microinjected it into quiescent vascular smooth muscle cells. To determine the effect of the Gax protein on serum-induced cell proliferation, the effect of GST-Gax protein was compared to the effect of known protein regulators of cell growth.

To produce the Gax protein evaluated herein, the cDNA coding regions for Gax was fused in frame to the pGEX-2T expression vector obtained from Pharmacia Biotechnology, and then expressed in *E. coli*. Specifically, GST-Gax was produced according to the following procedure: the coding region of Gax cDNA spanning from nucleotides 200-1108 was amplified by polymerase chain reaction methods using the following primers:

5'GCGCGCGTCGACGAACACCCCCTCTTTGGC 3' and

5'GCGCGCAAGCTTTCATAAGTGTGCGTGCTC 3'

The resulting DNA was digested with SalI and HindIII restriction enzymes and cloned into SalI and HindIII sites in the polylinker of pGEM3-1T in vitro transcription translation vector described in Patel R.C. and Sen G.C. (1992) "Identification of the Double-stranded RNA-binding Domain in the Human Interferon-inducible Protein Kinase," J. Biol. Chem. Vol. 267; pp. 7671-7676. The BamHI to NaeI fragment of pGEM3-1T containing the Gax coding region was then sub-cloned into the same sites of pGEX-2T. The pGEX-2T vector with the YY1 cDNA, used to

produce GST-YY1, was from Thomas Shenk at Princeton University.

The resultant glutathione S-transferase fusion proteins were purified by affinity chromatography on glutathione-agarose beads. E. coli XL1-blue cells were then transformed with the appropriate plasmid and were grown to a density of 0.6-0.8 A_{600} and induced with 0.5 mM isopropyl-B-D-thiogalectopyrenoside for 2 hours. The cells were harvested and lysed by ultrasonic vibration in phosphate buffered saline containing 1% triton x-100, 1mM PMSF and 5 μg/ml aprotinin. The lysate was centrifuged at 15,000xg and the supernatant was collected. The supernatant was bound to the glutathione sepharose from Pharmacia (0.5 ml of resin per 100ml of bacterial culture) for 2 hours on a rotator at 25 rpm. The slurry was pelleted by centrifugation at 1000xg for 2 minutes, then washed twice with complete lysis buffer then washed twice with lysis buffer lacking triton x-100. The bound protein was eluated for 30 minutes with phosphate buffered saline containing 10 mM reduced glutathione, from Sigma Chemical Company, 40 mM DTT and 150 mM NaCl. Purity of the GST-Gax protein was greater than 90% as determined by SDS-PAGE gels stained with Coomassie blue.

To produce recombinant MHox, its cDNA was fused in frame to the pQE-9 *E. coli* expression vector obtained from Qiagen, Inc., Chatsworth, CA, then expressed in bacteria, and purified by adsorption to a nickel column.

For microinjection, proteins were concentrated in a buffer containing of 20 mM Tris, 40 mM KCl, 0.1 mM EDTA, 1 mM β-mercaptoethanol, and 2% glycerol using Centricon-30 from Amicon microconcentrators. Concentrated proteins were stored in this buffer in aliquots at -80° C.

Microinjection and Cell Culture Methods

Microinjections were performed using a semiautomatic microinjection system from Eppendorf Inc. in conjunction with a Nikon Diaphot phase contrast microscope. According to Peperkole, R., et al. (1988) Proc. Natl. Acad.

Sci. USA Vol. 85, pp. 6758-6752, The injection pressure was set at 70-200 hPa and the injection time was 0.3 to 0.6 seconds.

After injection, cells were stimulated 24 hours with medium containing 10% fetal calf serum, and the incorporation of 5'-bromo-2'-deoxyuridine, hereinafter also referred to as "BrdU" was measured with a cell proliferation kit according to the directions of its manufacturer, Amersham. When fetal calf serum-stimulated BrdU labeling was determined, BrdU was included for 24 hours with the medium used to stimulate the cells. Where the ability of microinjected proteins to stimulate growth in serum-poor medium was measured, cells were incubated 24 hours in the same low serum medium used to induce quiescence, but supplemented with BrdU. After labeling, the cells were fixed with acid-ethanol, and the percentage of nuclei positive for BrdU uptake was determined for protein-injected and buffer-injected cells. The percent of cell growth inhibition was calculated according to the following formula:

$$\%Inhibition = \frac{\frac{CL}{CT} - \frac{IL}{IT}}{\frac{CL}{CT}} x100$$

where IL represents the number of injected labeling positive for BrdU; IT, the total number of injected cells; CL the number of control-injected cells labeling with BrdU; CT, the total number of control-injected cells counted. With this equation, inhibition of mitogen-induced entry into S phase is represented by a positive number and stimulation of cell growth is represented by a negative number.

Evaluation of Gax Protein

To determine if the Gax protein inhibits the entry of mitogen stimulated vascular smooth muscle cells into S-phase, the effect of the Gax protein was compared to

proteins known to effect cell proliferation, and to control proteins. Such comparison proteins include a neutralizing antibody against *ras*, "Y13-259," which is highly effective in blocking S phase entry when microinjected into NIH3T3 cells; the transcription factor MHox, a homeodomain protein unlikely to have an inhibitory effect on cell proliferation; and YY1, a zinc finger transcription factor unlikely to have a negative effect on cell growth.

Quiescent rat vascular smooth muscle cells were microinjected with either 0.6 mg/ml GST-Gax protein; 1.6 mg/ml MHox; 1.2 mg/ml YY1; 8 mg/ml Y13-259; 2mg/ml GST alone; or 8 mg/ml mouse anti-human IgG. The cells were then stimulated for 24 hours with 10 % fetal calf serum in medium containing BrdU. After 24 hours, the fraction of nuclei labeling with BrdU was determined and percentage inhibition of S-phase entry calculated. The results are summarized in Table 2.

TABLE 2
Effect of Microinjected Proteins on the Serum-induced Proliferation of Vascular Smooth Muscle Cells

Treatment	Number of Experiments	Total Number of Cells Examined	Mean % Inhibition of FCS-stimulated Growth ± Standard Error
Antibody Y13-259	2	328	60.8 ± 3.9
Mouse anti-human IgG	3	330	-3.4 ± 4.5
GST-Gax	15	2943	42.7 ± 3.3
MHox	2	236	-5.3 ± 9.3
GST-YY1	5	306	0.0 ± 12.2
GST	7	1144	-2.6 ± 2.1

FCS - fetal calf serum

BrdU labeling of quiescent vascular smooth muscle cells was 10.1 ± 1.2% (N=12, total number of cells counted = 2659); for uninjected FCS-stimulated vascular smooth muscle cells, 54.8 ± 2.4% (N=27, total number of cells counted = 4282); and for sham-injected FCS-stimulated cells, 49.6 ± 2.5% (N=27, total number of cells injected = 3401).

As shown in Table 2, the GST-Gax protein

inhibited vascular smooth muscle cell entry into S-phase by 42.7%. The GST Gax protein effect on mitogen-stimulated entry into S phase is specific. The other injected proteins GST, YY1, MHox and the mouse anti-human IgG failed to inhibit vascular smooth muscle cell growth. In comparison to the GST-Gax protein, the antibody Y13-259, as anticipated, significantly decreased mitogen-induced cell proliferation. Vascular smooth muscle cells microinjected with Y13259 demonstrated a 61 ± 4% decrease in cell entry into S-phase

Gax Protein Inhibits Vascular Smooth Muscle Cell Proliferation in a Dose-Dependent Manner.

To determine the concentration of microinjected GST-Gax required to inhibit vascular smooth muscle cell growth, solutions containing different concentrations of GST-Gax protein were microinjected into quiescent vascular smooth muscle cell and the effects on mitogen-stimulated entry into S phase examined. Specifically, vascular smooth muscle cells were rendered quiescent by incubation in medium containing 0.5% calf serum for three days. The cells were microinjected with varying concentrations of GST-Gax, and stimulated with 10% fetal calf serum, and labeled with BrdU. After 24 hours, the percentage inhibition of cell proliferation was determined. Each data point represents the mean ± standard error of 3-5 experiments in which 100-200 cells per experimental group were injected.

As shown in Figure 11, the cellular growth inhibition by the GST-Gax protein is dose dependent. Little or no growth inhibition was observed when 0.2 mg/ml GST-Gax protein was injected. The maximal growth inhibition was obtained with approximately 0.5 mg/ml of the GST-Gax protein.

Gax Inhibits Proliferation of Several Cell Types

To determine whether the GST-Gax protein inhibits growth in other cells types, the GST-Gax protein was microinjected into quiescent SV40-transformed vascular smooth muscle cells, BALBc3T3 cells, NIH3T3 cells, human vascular smooth muscle cells, and human fibroblasts. The SV40 transformed cell line was derived from rat vascular smooth muscle cells transformed with the SV40 large T antigen. These cells, while immortalized, retain many differentiated characteristics of untransformed vascular smooth muscle cells. The cells were microinjected with either 0.6 mg/ml GST-Gax protein or 2 mg/ml GST were then stimulated with 10% fetal calf serum, and labeled for 24 hours with BrdU. The results are shown in Table 3.

TABLE 3

EFFECT OF MICROINJECTED GST-GAX PROTEIN ON CELL PROLIFERATION IN DIFFERENT CELL TYPES

Cell type	GST-GAX protein	Number of Experiments	Number of Cells Examined	Mean & Range Inhibition of FCS-Stimulated Growth	Mitotic Index in Response to FCS
SV40-transformed VSMC	Yes	4	448	27.2 ± 2.0	--
SV40-transformed VSMC	No			N/A	0.60 ± 0.02
BALB/c 3T3 cells	Yes	4	464	30.5 ± 10.9	--
BALB/c 3T3 cells	No			N/A	0.64 ± 0.03
NIH3T3 cells	Yes	4	420	23.2 ± 1.8	--
NIH3T3 cells	No			N/A	0.70 ± 0.02
Human VSMC	Yes	3	506	46.6 ± 8.1	--
Human VSMC	No			N/A	0.33 ± 0.02
Human fibroblasts	Yes	3	336	44.5 ± 2.1	--
Human fibroblasts	No			N/A	0.36 ± 0.01

FCS - fetal calf serum
VSMC - vascular smooth muscle cells

SV40 - transformed vascular smooth muscle cell proliferation was inhibited by GST-Gax protein, as shown in Table 3. The GST-Gax protein also inhibited the proliferation of fibroblast cell lines NIH3T3 and BALB/c 3T3. GST-Gax protein also inhibited the proliferation of human cells, specifically human vascular smooth muscle cells and human foreskin fibroblasts. These results indicate that Gax action is not cell type-specific, although there are differences in the extent inhibition

among the different cell types. The Among the human cells, the GST-Gax protein exhibits maximal inhibition in vascular smooth muscle cells, the cell type in which the Gax gene is normally expressed. Similarly among the rat cells, the GST-Gax protein exhibits maximal inhibition in vascular smooth muscle cells, the cell type in which the Gax gene is normally expressed.

An Oncogenic Ras Protein Can Reverse Growth Inhibition Caused by the Gax protein

To characterize the mechanism of the growth inhibition conferred by the GST-Gax protein, the effects of GST-Gax protein and the transforming oncoprotein, the *ras* mutant Ras(Leu-61) were compared by microinjecting these proteins into rat vascular smooth muscle cells. A solution containing both 0.5 mg/ml GST-Gax protein and 0.5 mg/ml Ras(Leu-61) was microinjected into quiescent vascular smooth muscle cells. For comparison, other vascular smooth muscle cells received either 0.5 mg/ml GST-Gax protein or 0.5 mg/ml Ras(Leu-61) or 0.5 mg/ml GST. The injected cells were then incubated for 24 hours with medium containing 10% fetal calf serum and BrdU. The results are shown in Figure 12.

As shown in Figure 12, when Ras(Leu-61) alone was injected, there was an increase in BrdU-labeling as compared to both control-injected cells. In cells injected with GST-Gax protein, growth was inhibited 39%. When the GST-Gax protein and Ras(Leu-61) were coinjected in the cells, the Ras(Leu-61) reversed the growth inhibitory effects of the GST-Gax protein, and the percentage of cells staining positive for BrdU in cells receiving both the Ras(Leu-61) and GST-Gax protein were nearly identical to that observed in cells receiving just the Ras(Leu-61). Thus, the Ras oncoprotein completely reversed the effect of the GST-Gax protein establishing that the presence of GST-Gax protein is not toxic to cells.

The Gax Protein Inhibits Cell Growth when Microinjected Before the GI to S Boundary

To determine the point in the cell cycle when the Gax gene exerts its growth inhibitory effects, the time of S phase onset was determined in rat vascular smooth muscle cells. The vascular smooth muscle cells were stimulated with 10% fetal calf serum and pulse labeled with 10 mCi/ml ^{3}H-thymidine for one hour at different times after stimulation. Serarate cultures of the cells were microinjected with GST-Gax protein at various times after receiving 10% fetal calf serum and labeled with BrdU between 10 and 24 hours after receiving the fetal calf serum. Percent inhibition of S-phase entry was determined at each time point. The results are shown in Figure 13.

As shown in Figure 13, S phase onset, indicated by the uptake of ^{3}H-thymidine, occured at approximately 16-18 hours after mitogen stimulation. GST-Gax protein significantly inhibited vascular smooth muscle cell entry into the S phase when microinjected at any time from stimulation up until approximately 12 hours. However, GST-Gax protein was ineffective when injected at 15 hours. Thus it appears that the Gax gene inhibits a critical step in cell cycle progression prior to the G_1/S boundary; perhaps before the restriction point in G_1 where eukaryotic cells are irreversibly committed to entering the S phase.

Gax Gene Expression is Rapidly Down Regulated in Vivo Upon Acute Blood Vessel Injury

The Gax gene expression in normal blood vessels and in injured blood vessels was compared to determine whether Gax gene down-regulation occurs in response to injury-induced smooth muscle cell proliferation in vivo. Adult male Sprague-Dawley rats were subject to acute vessel injury by balloon de-endothelialization in the carotid arteries according to the methods of Majesky, M.W., et al. J. Cell. Biol. (1990) Vol. 111, pp. 2149-2158. The expression levels of Gax, that is, the mRNA levels, were

assessed relative to that of glyceraldehyde 3-phosphate dehydrogenase (hereinafter also referred to as "G3") by a quantitative polymerase chain reaction. At various times following balloon de-endothelialization the rats were sacrificed and the total RNA was isolated from the vascular smooth muscle tissues using the TRI reagent from Molecular Research Center, Inc. The cDNA was synthesized from the extracted RNA with MMLV reverse transcriptase from Bethesda Research Labs. Aliquots of the cDNA pools were subjected to polymerase chain reaction amplification with AmpliTaq DNA polymerase from Perkin-Elmer in the presence of α32P-dCTP with the following cycle conditions: 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. The final cycle had an elongation step at 72°C for 5 minutes. The primers for the rat Gax amplification were: 5'-CCCGCGCGGCTTTTACATTAGGAGT-3' and 5'-GCTGGCAAACATGCCCTCCTCATTG-3'. The primers for the rat G3 gene were 5'- TGATGGCATGGACTGTGGTCATGA-3' and 5'-TGATGGCATGGACTGTGGTCATGA-3'. The Gax cDNA was amplified for 30 cycles, and G3 was amplified for 25 cycles in the same reaction vessels. The amount of a radioactive label incorporated into the amplified cDNA and G3 fragments was determined by subjecting the fragments to electrophoresis on a 1% agarose gel, then excising the bands and liquid scintillation counting. Since the mRNA levels of glyceraldehyde 3-phosphate dehydrogenase remain relatively constant following this procedure (see J.M. Miano et al. 1990, Am. J. Path. 137, 761-765), the ratio of radiolabel incorporation into the Gax-derived amplified bands and the G3-derived amplified bands corrects for differences arising from the efficiency of RNA extraction from the different animals, and it provides a measure of Gax mRNA levels in the normal and injured vascular tissues. These ratios are plotted in Figure 14.

As shown in Figure 14, the Gax mRNA expression was down-regulated in response to acute vessel injury by as much as a factor of 20. This down-regulation was rapid and

appeared complete by 2 hours, the first time-point following the de-endothelialization procedure. Collectively, these data corroborate the Gax gene down-regulation in cultures of vascular smooth muscle cells following growth factor stimulation. Further, these data show that Gax gene expression is an early marker of the cell cycle activity associated with the initiation of vascular restenosis, and they indicate that Gax has a regulatory role following blood vessel injury.

The present invention includes: the DNA sequences encoding a protein, or portion thereof, which inhibits vascular smooth muscle cell proliferation; the messenger RNA transcript of such DNA sequence; and an isolated protein which inhibits vascular smooth muscle cell growth.

For example, the DNA sequences include: DNA molecules which, but for the degeneracy of the genetic code would hybridize to DNA encoding the Gax protein, thus the degenerate DNA which encodes the Gax protein; DNA strands complementary to DNA sequences encoding the Gax protein or portions thereof including DNA in Figures 1 and 3 or portions thereof; heterologous DNA having substantial sequence homology to the DNA encoding the Gax protein, including the DNA sequences in Figures 1 and 3 or portions thereof.

The isolated protein includes, for example, portions of the Gax protein; the Gax protein of animals other than rat and human; and proteins or portions thereof having substantially the same amino acid sequence as shown in Figures 1 and 3 or portions thereof.